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We hypothesize that components of DNA-related checkpoint pathways in addition to members of the ATM protein/lipid kinase family are conserved in all eukaryotes. This is based on functional similarities in the pathways and the conservation between evolutionarily disparate budding and fission yeasts. Work described herein discusses a new model for ATM-dependent regulation c the effector kinase Chk2. We show that DNA damage induces oligomerization of Chk2. Chk2 dimerization can be mediated by an interaction between an FHA domain on one Chk2 molecule and a phosphopeptide on another. The signficance of this interaction fo DNA damage-dependent regulation of Chk2 is discussed.

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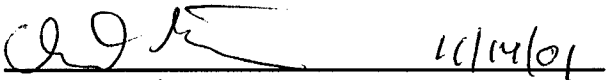

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5) INTRODUCTION

We originally hypothesized that components of DNA-related checkpoint pathways in addition to members of the *ATM* protein/lipid kinase family are conserved in all eukaryotes. This is based on functional similarities in the pathways and the conservation between the evolutionarily disparate budding and fission yeasts. Our goal was to identify additional regulators of mammalian DNA checkpoints by virtue of structural and functional homology with known checkpoint genes in budding yeast. We had proposed to use both structural and functional screens to identify human homologs of yeast damage checkpoint proteins Rad53 and Rad9. Once identified, such components would be ordered into pathways for mammalian checkpoint function, with emphasis on p53 regulation, cell cycle regulation, and complementation of *ATM* defects.

	Saccharomyces cerevisiae	*Schizosaccharomyces pombe*	*Homo sapiens*	
	Mec1 Tel1	rad3#	Atr Atm	PI kinase family; protein kinase
	Ddc2?	rad26 (*P)#		
	DAMAGE PATHWAY			
	Rad9 (*P)	Crb2/Rhp9(*P)	Brca1? p53BP1? Nibrin? KIAA0170?	**BRCT** domains
	Rad24	Rad17#	Rad17 (RFC4?)	
	Mec3/Pip3			
		Hus1# *P	**Hus1**	
	Rad17	**Rad1#**	**rad1**	
	Ddc1 (*P)	**Rad9 #**	**Rad9**	
	DPB11	cut5		**BRCT** domains replication upstream Rad53
	Sld2/DRC1			replication upstream Rad53
	POL2		Pol	replication upstream Rad53
	RFC5		RFC	replication
	PRI4		Pol	replication Polα-Primase downstream Rad53
	Rad53 *P	cds1 *P	cds1/Chk2*P	protein kinase **FHA** domain(s)
	Chk1	Chk1*P	Chk1*P	protein kinase

Table 1. Conservation of yeast and mammalian DNA checkpoint genes. *P indicates that damage induces protein phosphorylation.

(6) BODY

The major objective for this work has now shifted from identification of mammalian DNA checkpoint genes that are homologs of yeast genes, to their characterization. As we hypothesized, the major components of DNA checkpoint pathways have been shown to be conserved between budding yeast and humans. At the time of submission of the original proposal, the only human ortholog of a yeast checkpoint gene was Atm. Since then, this list has been extended to the genes listed below, which includes a homolog of yeast Rad53, which we originally sought (Table 1). With the identification of the yeast Rad53 homolog, Chk2/Cds1 [1-4], we have now begun to investigate how Chk2 is regulated, and how it regulates targets. This work is directly relevant to breast cancer, since it is now clear that Chk2 is an intermediary linking DNA checkpoint pathways from Atm to p53; since Chk2 phosphorylates and modulates Brca1 function [5], and since Chk2 mutations are found in variant p53+ forms of Li-Fraumeni syndrome, which predisposes to breast cancer and other cancers [6].

Connection with Approved Technical Objectives.

Technical Objective 1. Protein-interaction screens for mammalian Rad53 and Rad9 homologs.
Technical Objective 2. Cloning of mammalian DNA checkpoint genes by complementation of defects in yeast.
Technical Objective 3. Screening based upon protein sequence homology.
Technical Objective 4. Characterization of genes in mammalian cells.

As described in earlier annual reports, we had already made substantial progress on Objectives 1 and 3. Objectives 1,2, and 3, which were various approaches to identifying mammalian homologs of Rad53 and Rad9 were made partially superfluous by the discovery of the Rad53 homolog Chk2. Continued work on Rad9 homologs and on Objective 4, is the subject of the present and future work. This ongoing work is divided into two components, upstream regulators and effectors. The work is being performed by two postdoctoral fellows, Lyuben Tsvetkov and Xingxhi Xu.

Mechanisms of Chk2 activation in response to DNA damage

In *S. cerevisiae*, *MEC1* and *TEL1* are functionally and structurally related to the human tumor suppressor *ATM*. *MEC1* and *RAD53*, two essential genes, play a central role in DNA damage checkpoints at all cell cycle stages. Our lab showed that Rad9 is a regulator coupling DNA damage to signaling through Mec1 to activate Rad53 and Chk1 in G1 and G2 checkpoints but not in the S-phase checkpoint [7, 8]. Phosphorylation of both Rad53 and Rad9 in response to DNA damage is *MEC1*-dependent.

Similarly, in mammals, activation of Chk2 requires Atm and/or Atr, the orthologs of Mec1 and Tel1. Double-stranded DNA breaks activate Atm, which in turn phosphorylates Chk2 at multiple sites, including T68 [1]. Substitution of T68 with other amino acids impairs activation of Chk2.

Cell-free activation of Chk2.

Although it is clear that Atm or Atr are required for DNA damage-dependent activation of Chk2, it has not been possible to activate Chk2 or its yeast homologs by phosphorylation with either of these two enzymes or their yeast equivalents. A fortuitous observation in our laboratory has now paved the way for cell-free activation of Chk2 in a mammalian system. We found that Chk2 produced by coupled transcription/translation in a reticulocyte lysate system was phosphorylated. Phosphorylation of rabbit reticulocyte Chk2 occurs even with kinase-

defective Chk2, meaning that transphosphorylation occurs in this cell-free system. Based upon caffeine and wortmannin sensitivity patterns, it appears that rabbit Atm is required for phosphorylation of Chk2 produced in vitro.

In contrast to Chk2 produced by translation in reticulocyte lysate, Chk2 produced by translation in a wheat germ system was not hyperphosphorylated. Importantly, this lack of phosphorylation correlated with weak in vitro kinase activity. However, incubation of wheat germ-produced Chk2 with reticulocyte lysate enabled Chk2 phosphorylation and catalytic activation. To our knowledge, this is the first mammalian cell-free system to enable catalytic activation of Chk2. Further work with this in vitro activation system will make it possible for us to determine the minimal components necessary for activation of this protein.

Chk2 regulation by oligomerization.

Earlier unpublished work in our laboratory had shown that the yeast Chk2 homolog Rad53 can interact with itself in two hybrids screens. This suggested that Chk2 oligomerization may be an important mechanism for Chk2 activation. To determine if this was the case, we measured oligomerization of Chk2 using a variety of in vivo and in vitro assays. For example, FLAG-tagged and HA-tagged Chk2 were coimmunoprecipitated when expressed in 293 cells. Importantly, the association was enhanced by DNA damage, suggesting that it is functionally relevant for the DNA damage response.

Chk2 has a N-terminal SQ/TQ cluster followed by a FHA (forkhead-associated) domain, and a C-terminal kinase domain. It differs from yeast Rad53 in that the latter (uniquely among FHA-containing protein kinases) has a second FHA domain. The Chk2 SQ/TQ cluster contains seven potential phosphorylation sites for Atm. Threonine 68 is one major Atm phosphorylation site in response to ionizing irradiation [1]. Earlier work in our lab showed that FHA domain-containing proteins mediate interactions with phosphoproteins through FHA domains, based on the phospho-Rad9/Rad53 interaction [8].

Since we had determined that DNA damage induces association of Chk2 in a complex containing other Chk2 molecules, we wondered whether interactions between Chk2 FHA domains and phospho-SQ/TQ clusters were involved. We found that for in vivo co-immunoprecipitation experiments, at least one molecule must have an FHA domain, and the other molecule must have the SQ/TQ cluster. GST-tagged Chk2 FHA domain will pull down Chk2 from DNA damaged cells, also suggesting that DNA damage-dependent phosphorylation of Chk2 promotes Chk2 oligomerization. Although these data do not preclude indirect interaction of Chk2 molecules within larger checkpoint complexes, we found that bacterially expressed forms of Chk2 will interact. When WT Chk2 is produced at high concentrations in bacteria, it undergoes partial autophosphorylation, including phosphorylation at the *trans* site T68 favored by Atm. Bacterially-produced GST-FHA domain will pull this protein down, demonstrating that homotypic interaction can occur between the Chk2 FHA domain and autophosphorylated Chk2 in the absence of other eukaryotic proteins. Finally, interactions between bacterially expressed kinase defective Chk2 (non-phosphorylated) and a His-Flag-tagged WT Chk2 (autophosphorylated) were abolished by pretreatment with lambda phosphatase. Hence, these homotypic interactions are indeed phosphorylation-dependent.

An important function of Chk2 oligomerization may be that it enables cross-phosphorylation of Ch2k molecules, leading, in turn, to full activation of Chk2. We found, using in vitro kinase assays containing bacterially produced kinase-defective GST-Chk2 and WT His-

FLAG-Chk2, that transphosphorylation of the kinase-defective molecule could occur, including phosphorylation at T68.

In summary, we have shown that DNA damage can induce oligomerization of Chk2 in vivo, and that such complexes require both Chk2 FHA domains and phosphorylated Chk2 SQ/TQ clusters. The interactions are phosphorylation-dependent, and can be enabled by Chk2 autophosphorylation at T68, a major trans-regulatory target for Atm. We conclude that Atm-dependent phosphorylation of Chk2 may be a priming event that permits further autophosphorylation and dimerization of Chk2. Once activated, Chk2 may be able to activate other quiescent Chk2 molecules by transphosphorylation at T68, *even in the absence of functional Atm*. These phospho-Chk2 molecules may also assemble into oligomers, in large signaling complexes. A manuscript describing this work has been submitted for publication [11].

Chk2 and RPA

As we discussed in last year's report, one of the sources for potential Chk2 substrates is a subset of proteins that undergo ATM-dependent phosphorylation after exposure of cells to IR. One of those proteins is the middle subunit of replication protein A (Rpa2). We have performed a kinase assay with GST-Chk2 and Rpa, generously provided by Bruce Stillman's laboratory. We have found that GST-Chk2 phosphorylates Rpa1 and Rpa2, but GST-Chk2-KD does not. We have investigated by co-immunoprecipitation if Rpa and Chk2 can interact. We have found that a small subpopulation of RPA2 co-immunoprecipitates with HA-Chk2 by IP with anti-Chk2 and anti-HA antibodies.

To determine if Rpa2 phosphorylation after DNA damage is Chk2-dependent we have performed several experiments. Treatment of HT-1080 cells with 1 mM camptothecin (CPT), a topoisomerase I inhibitor, for 1 hour causes Rpa2 hyperphosphorylation, which can be prevented by 15min pre-treatment of cells with 5mM a protein kinase inhibitor UCN-01 (7-hydroxystaurosporine). The concentration of UCN-01that prevents Rpa2 phosphorylation is quite different from the one that inhibits Chk1 (IC50=15nM). Also, we have found that treatment of HT-1080 cells with 1mM CPT causes gel mobility shift of Chk2, which can be diminishes by pre-treatment of cells with 1-5mM UCN-01.

Experiments with pre-treatment of HT-1080 cells, stably transfected with pcDNA3-HA-Chk2, with different concentrations of UCN-01 showed that dose/ response curves of Rpa2 and Chk2 phosphorylation in response to treatment with 1mM CPT are similar. To determine if the Rpa2 and Chk2 phosphorylation are ATM-dependent, we transiently transfected AT cells with pcDNA3-HA-Chk2 and treated those cells with CPT. Chk2 and RPA2 proteins were shifted, so we concluded that those responses are *ATM*-independent. Other possible candidate kinases are Atr and DNA-PK, which we can distinguish by treatment of AT cells with different concentration of protein kinase inhibitors caffeine and wortmannin. We have pre-treated AT cells, transiently transfected with HA-Chk2 plasmid, with 10m wortmannin or 3mM caffeine for 20 min, and treated later with 1mM CPT. Chk2 and Rpa2 phosphorylation was caffeine-sensitive and wortmannin-insensitive, so most probably this pathway goes through Atr rather than DNA-PK.

To determine if Rpa2 phosphorylation is Chk2-dependent we treated HT-1080 cells, stably transfected with HA-Chk2 and HA-Chk2D368A (KD-dominant negative mutant), with CPT and then measured Rpa2 hyperphosphorylation (mobility shift in the gel) by immunoblotting. We did not detect a decrease of Rpa2 hyperphosphorylation in cells stably transfected with dominant-negative Chk2 mutant.

In summary, we have found that Chk2 can phosphorylate the large and middle subunits of Rpa *in vitro*. Also, we have observed a physical interaction between Chk2 and Rpa2 by co-immunoprecipitation. The data associating phosphorylation of Chk2 and Rpa2 after treatment with CPT are correlative only and it will be necessary to establish a better system to verify the Chk2-dependence of Rpa2 phosphorylation.

(7) KEY RESEARCH ACCOMPLISHMENTS.

Our work lends further credence, but not proof, to the hypothesis that RPA is a substrate for Chk2 in vivo. Our work has identified a novel ability of Chk2 to phosphorylate itself at T68, and to form dimers as a result of this autophosphorylation. On this basis, we have proposed a novel model for Chk2 activation:

- Chk2 produced in wheat germ extracts can be activated in vitro in a cell-free system, with Atm being important.
- Chk2 autophosphorylates on a major site known to be important for trans-regulation by Atm.
- Autophosphorylation of the Chk2 SQ/TQ cluster enables formation of Chk2 oligomers mediated by phosphorylation dependent binding to the Chk2 FHA domain.
- Chk2 undergoes damage-dependent oligomerization in vivo.
- In vitro phosphorylation of Rpa1 and Rpa2 by Chk2.
- Correlations between phosphorylation of chk2 and RPA after CPT treatment.

(8) REPORTABLE OUTCOMES.

Work on Chk2 dimerization and activation has been submitted for publication to Molecular and Cellular Biology:

Xu, X., Tsvetkov, LM., and **Stern**, D.F. Chk2 activation and phosphorylation-dependent oligomerization.

(9) CONCLUSIONS

This proposal hypothesized that there would be a mammalian homolog of Rad53, and that this homolog would be important in regulation of DNA checkpoint pathways. The human homolog was discovered three years ago, and in the previous cycle we developed systems and reagents for expression and analysis of all of the pertinent proteins, and are made headway on association of Chk2 with DNA damage response complexes including FHA domain-containing proteins.

Work on these genes and pathways is not simply an academic exercise. These DNA checkpoint proteins have important roles in breast cancer. Although no clear Rad9 ortholog has been identified, it does share a protein homology domain and, possibly, functionality, with the breast cancer tumor suppressor gene *BRCA1*. Mutations in the *MEC1* ortholog *ATM* are apparently responsible for a significant fraction of hereditary breast cancers. The *RAD53* ortholog *CHK2/CDS1* regulates breast cancer tumor suppressor proteins p53 and Brca1. Also, mutations in *CHK2* are responsible for a variant form of the breast cancer predisposing Li-Fraumeni syndrome, in which *TP53* is not mutated. Not only will progress on these problems enhance our understanding of carcinogenic processes, they may be important for optimization of breast cancer therapies. The response of tumor cells to genotoxic chemotherapeutic agents is

greatly affected by the affects they have on the DNA checkpoint pathways including Chk2. It can be anticipated that fruits of this work will include better therapeutic modulators, and the ability to maximize utility of existing therapies.

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Chk2 Activation and Phosphorylation-Dependent Oligomerization

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Abstract

The tumor suppressor gene *CHK2* encodes a versatile effector serine/threonine kinase involved in responses to DNA damage. Chk2 has an amino-terminal SQ/TQ cluster domain (SCD) followed by a forkhead-associated (FHA) domain, and a carboxyl-terminal kinase catalytic domain. Mutations in the SCD or FHA domain impair Chk2 checkpoint function. We show here that autophosphorylation of Chk2 produced in a cell-free system requires transphosphorylation by a wortmannin-sensitive kinase (probably ATM and/or ATR). Both SQ/TQ sites and non-SQ/TQ sites in the Chk2 SCD can be phosphorylated by active Chk2 itself. Amino acid substitutions in SCD and the FHA domains impair *auto-* and *trans*-kinase activities of Chk2. Chk2 forms oligomers that minimally require the FHA domain of one Chk2 molecule and the SCD within another Chk2 molecule. Chk2 oligomerization *in vivo* increases after DNA damage, and, if the damage is induced by gamma irradiation, this increase requires *ATM*. Chk2 oligomerization can occur in the absence of other eukaryotic proteins, and depends upon phosphorylation. Chk2 can cross-phosphorylate another Chk2 molecule in a complex. These results suggest that Chk2 oligomerization regulates Chk2 activation, signal amplification and transduction of the DNA damage checkpoint pathways.

Introduction

DNA damage activates response pathways that halt the cell cycle, induce the transcription of genes that facilitate DNA repair and DNA replication, alter telomeres, and induce apoptosis if damage cannot be repaired (54). Checkpoint defects may result in genomic instability, a mutagenic condition that predisposes organisms to cancer. On the other hand, DNA damaging agents, in the form of gamma irradiation and genotoxic drugs, are mainstays of current cancer treatment regimens. Manipulation of checkpoint genes may ultimately benefit chemo- and radiotherapy (18).

Checkpoint pathways are analogous to growth factor-regulated signal transduction pathways, in which DNA damage initiates a signal that is transduced and amplified to generate checkpoint responses. Although the precise nature of the initial step of signal transduction is poorly understood, damaged DNA activates a cascade of protein kinases. In mammals, these kinases includes phospho-inositide kinase (PIK)-related proteins Atm (Ataxia Telangiectasia Mutated) and Atr (Atm and Rad3-related), and the downstream serine/threonine checkpoint kinases Chk1 and Chk2. Orthologs of these genes have been identified in yeasts, with *Saccharomyces cerevisiae* (*S.c.*) Mec1 and Tel1 serving Atm or Atr-like functions, and Chk1 and Rad53 resembling mammalian Chk1 and Chk2, respectively. Effectors that execute the functions of the DNA damage responses include substrates of both PIK and CHK kinases.

Atm is a central signaling protein in the response to ionizing radiation (IR) and other sources of double-strand DNA breaks. Homozygous mutations in *ATM* are responsible for the pleiotropic Ataxia-Telangiectasia (A-T) syndrome, which includes cancer predisposition and sensitivity to ionizing radiation along with progressive

cerebellar defects (24). Chk2 is a major effector of Atm (5, 6, 9, 31). Both the breast cancer susceptibility gene product Brca1 (12, 27) and p53 (3, 7, 10, 20, 42) are substrates of Atm and Chk2. Li-Fraumeni syndrome (LFS) is a hereditary disorder predisposing to multiple neoplasms and is generally associated with constitutional *TP53* mutation. *CHK2* mutations have been identified in some LFS kindreds that do not have p53 mutations (4, 48), in myelodysplastic syndromes and acute myeloid leukemias (22), and in small cell lung cancer (19). *CHK2*, therefore, is a regulator of tumor suppressor gene products and is itself a likely tumor suppressor gene

Chk2 activation in response to ionizing irradiation, is *ATM*-dependent (5, 6, 9, 31). Activated Chk2 in turn phosphorylates p53 at Serine-20 (10, 20, 42), Cdc25A at Serine-123 (16), and Cdc25C at Serine-216 (5, 6, 9, 31), contributing to G1/S, S and G2/M checkpoints respectively.

Atm-like PIKs show a strong sequence preference for phosphorylation of SQ/TQ sites (2, 25). The SCD, near the amino terminus of Chk2, includes seven SQ/TQ sites, including known Atm- and Atr-dependent phosphorylation targets (1, 32, 33, 53). The SCD is followed by a FHA (forkhead-associated) domain, and a carboxyl-terminal kinase catalytic domain. Activation of Chk2 occurs through a series of steps, including trans-phosphorylation by Atm or Atr at sites within the SCD including T68 (1, 33, 53). PIK-dependent phosphorylation is required for autophosphorylation within the activation loop of the catalytic domain at T383 and/or T387 (26).

The intact FHA domain is required for damage-dependent activation of Chk2 (10, 26). FHA protein homology domains were first identified in a subset of forkhead transcription factors (21). They are present in a wide variety of proteins in prokaryotes

and eukaryotes (28). Many eukaryotic FHA domain-containing proteins are found in the nucleus and are involved in DNA repair and cell cycle arrest (28). Recent work shows that FHA domains are phosphopeptide recognition domains (13, 14, 45, 47). However, only a small number of protein/protein interactions that are mediated by FHA domains have been identified. They include association of an *Arabadopsis thaliani* phosphatase FHA domain with a phosphorylated receptor-like kinase (29, 45), and interaction of *S.c.* Rad53 with phosphorylated Rad9 (47), which operates upstream from Rad53 in the damage-dependent signaling cascade.

The FHA domain of the *S.c.* Chk2 homolog, Rad53, couples Rad53 to damage-dependent signals through direct binding to a second damage-response protein, Rad9 (15, 47, 49). DNA damage induces PIK-dependent phosphorylation of Rad9 (15, 49). Once phosphorylated, Rad9 binds tightly to the two FHA domains of Rad53 (13, 47). Disruption of this interaction either through mutations of Rad53 FHA domains (47), or mutations in the Rad9 site that binds Rad53 FHAs (M Schwartz and DF Stern, submitted for publication), prevents activation of Rad53. Since Rad9 and Rad53 both require Mec1 for activating phosphorylations, these results suggested that phospho-Rad9 acts as an adaptor that recruits Rad53 to an activating complex containing Mec1. Alternatively, it has been proposed that phosphorylated Rad9 dimer functions as a scaffold to bring Rad53 molecules into close proximity to each other, facilitating cross-phosphorylation between Rad53 molecules, and subsequent release of activated Rad53 (17).

Activation of protein kinases through regulated oligomerization has been demonstrated for both tyrosine kinases and serine/threonine kinases (23, 30, 41). We report here that Chk2 undergoes homo-oligomerization in response to DNA damage. This

process is mediated by the phosphorylated SCD in an activated Chk2 molecule and the FHA domain in another inactive Chk2 molecule. We propose that Chk2 oligomerization is central to regulation of Chk2 activation, signal transduction, and signal amplification.

Materials and Methods

Antibodies

Rabbit polyclonal anti- T26/S28 of Chk2 was a kind gift of Yi Tan (Cell Signaling Technology). This antibody recognizes Chk2 after gamma-irradiation, but not when T26 and S28 have been substituted with alanine (X Xu and DF Stern, unpublished data). Rabbit anti-phospho-T68 was produced by immunization with keyhole-limpet hemacyanin coupled to KSSLETVS-pTQELYSI. Mouse monoclonal anti-HA antibody (16B12) was purchased from BabCo/Covance; mouse monoclonal anti-Flag, rabbit anti-glutathione-S-transferease (GST), and mouse IgG from Sigma; horseradish peroxidase (HRP)-conjugated mouse anti-HA (12CA5) and rat anti-HA monoclonal antibodies were from Roche; and goat anti-Chk2 (N-17) from Santa Cruz. Antigen-antibody complexes were recovered with protein G plus protein A agarose (CalBiochem). HRP-conjugated secondary antibodies and chemiluminescence reagents were from Pierce.

Plasmids

A clone within the expressed sequence tag (EST) database (GenBank accession no. AA285249) containing the entire coding sequence of Chk2 was obtained from Thanos D. Halazonetis (Wistar Institute). For expression in mammalian cells, Chk2-coding sequences were amplified by polymerase chain reaction (PCR) and cloned into pcDNA3xHA-Neo and pcDNA3xFlag-Neo vectors, resulting in pcDNA-HAChk2 and pcDNA-FlagChk2. Point and internal deletion mutants were generated from pcDNA-HAChk2 using PCR-based site-directed mutagenesis (52). For expression in *Escherichia coli*, Chk2 sequences were cloned into pGEX4T vectors (Amersham Pharmacia Biotech) for glutathione-S-transferease (GST) fusions, and pTrcHis vectors (Invitrogen) for His-

tagged fusions. Human Cdc25C (aa200-256) was isolated by PCR from an EST clone (GenBank accession no. AW401554). Plasmid constructs were verified by sequence analysis. Primer sequences and detailed cloning strategies are available upon request. Wild type and kinase-defective (^{2870}D→A and ^{2875}N→K) Flag-*ATM* (7) constructs were kind gifts of Michael Kastan (St. Jude Children Hospital). pGEX-Chk2(1-222) and pGEX-Chk2(57-222) (8) were obtained from Susan Lees-Miller (University of Calgary).

Recombinant proteins

Expression of GST fusions or His fusions in strain DH5α was induced with 1mM IPTG (isopropylthio-β-D-galactoside) for 3-4 hours at 37°C. For GST fusion proteins, cell lysates were harvested in PBS (Dulbecco's phosphate-buffered saline lacking Ca^{++}/Mg^{++}) in the presence of 1 mg/ml of lysozyme and 10 units/ml of deoxyribonuclease I (DNase I). For His fusion proteins, cell lysates were collected in 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0, in the presence of 1 mg/ml of lysozyme and 10 units/ml of DNase I. Cell lysates then went through 10 cycles of freeze and thaw. GST- and His-fusion proteins were batch-purified using glutathione-sepharose beads (Amersham Pharmacia Biotech) or Ni-NTA beads (Qiagen), respectively according to the manufacturers' procedures. GST fusion proteins were eluted with 50 mM Tris, 10 mM glutathione, pH 8.0. His fusion proteins were eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0, and then dialyzed against PBS.

***In vitro* coupled transcription/translation assays**

Chk2 constructs (pcDNA series) were used as templates for *in vitro* transcription/translation of Chk2 in the absence or presence of [^{35}S]-Met-Cys labeling mix (Amersham Pharmacia Biotech). Promega TnT T7 Quick Coupled

Transcription/Translation reticulocyte lysate system and T7 Coupled Transcription/Translation wheat germ extract system were used in a standard 50-ul reaction according to procedures recommended by the manufacturer.

Cell culture and transfection

AT-deficient (GM5849C) Simian Virus 40-transformed human fibroblasts were obtained from Coriell Institute for Medical Research, Camden, N.J. Other cell lines were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U of penicillin/ml, 50 mg of streptomycin/ml. Transfection was performed using Fu-gene 6 (Roche) at a ratio of 1 ug plasmid: 2 ul Fu-gene. Stable transfectants were selected in medium containing G418 (Life Tech) at 700 ug/ml. Cells were treated with 1 mM of HU 24 hours after transfection for 24 hours. Cells were irradiated in a Mark I ^{137}Cs irradiator (Shepherd) at a dose rate of 1.8 Gy/min 48 hours after transfection. Cells were UV-irradiated at a dose of 50 J/m^2 using the Stratagene Crosslinker 48 hours after transfection.

Immunoprecipitation and immunoblotting

Cell lysate was harvested 2 hours after irradiation or 24 hours after HU treatment in TSD buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Sodium Deoxycholate, 0.1% Triton X-100, and protease inhibitor cocktail [Roche]). Two micrograms of antibodies were used for immunoprecipitation from 400 to 500 ug of total lysate at 4°C overnight. Precipitates were washed using TSD buffer lacking protease inhibitors. *In vitro* translation product was mixed with 300 ul of NETN (20 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail) for

immunoprecipitation. Precipitates were washed using NETN buffer lacking protease inhibitors. Immunoblots on nitrocellulose were blocked with 5% non-fat milk in PBST (0.5% Tween-20) and washed in PBST.

GST pull-down experiments

Two micrograms of soluble GST-fusion proteins and 20 ul of glutathione-sepharose beads were incubated with 500 ug of total lysate in TSD buffer derived from HEK293 cells expressing HA-tagged Chk2 and mutants (Figs. 6C, 7C, 7D), or with 0.5 ug of soluble wild type or kinase-defective His-Flag-Chk2 in the presence of 300 ul of NETN buffer (Figs. 6A, 6B, 8) at 4°C overnight. The beads were washed in NETN buffer lacking protease inhibitor.

***In vitro* kinase assays**

Mixtures of kinase (prepared as soluble GST- or His- fusion proteins, immune complexes, or GST affinity isolates) were incubated with substrates at 30°C for 5-10 min in 1X kinase buffer (20 mM Tris [pH 7.5], 10 mM $MgCl_2$,10 mM $MnCl_2$, 1 mM DTT] supplemented with either 2 uM nonradioactive ATP or 2 uM non-labeled and 10 uCi [γ-^{32}P]ATP (>5000 Ci/mmol, AA0018, Amersham Pharmacia Biotech).

Phosphatase treatment

Immunoprecipitates of HA-Chk2 produced by translation in the coupled reticulocyte lysate system were incubated with calf intestinal phosphatase (CIP, New England Biolabs) for 1 hour at 37°C. Soluble wild type His-Flag-Chk2 (0.5 ug) was incubated with 1000 units of λ phosphatase (New England Biolabs) in the presence of 2 mM $MnCl_2$ for 1-2 hours at 30°C in a 50-ul reaction volume.

Results

Cell-free system for activation of Chk2. In *S.c.*, DNA damage induces a stable modification of the Chk2 homolog Rad53 that results in elevated activity detectable by in gel kinase assays after denaturing gel electrophoresis and subsequent renaturation (35). This modification, probably phosphorylation, depends upon the Atm/Atr homolog Mec1. However, efforts to activate mammalian Chk2 *in vitro* by phosphorylation with Atm or DNA-dependent protein kinase (32)(X Xu and DF Stern, unpublished data) have been unsuccessful. Chk2 produced by coupled *in vitro* transcription/translation in rabbit reticulocyte lysates (RL) migrates heterogeneously in SDS-PAGE gels (Fig. 2A, lane 1). The slower migrating form was eliminated by phosphatase treatment (Fig. 2A, lanes 3 and 4). Expression of the kinase-defective D347A allele in RL yielded only the hypophosphorylated form (Fig. 2A, lane 2), indicating that autophosphorylation is reqired for the mobility shift. Phosphorylation of immunoreactive T26/S28 and T68 in the SCD was evident in both wild type and kinase-defective Chk2 derived from RL (Fig. 2B, lane 12). Thus, one or more protein kinases that can phosphorylate Chk2 at these sites are present in the RL. Since, the kinase-defective form does not undergo extreme mobility shift, (Fig. 2A, lane 2), phosphorylation at T26/S28 and/or T68 is not sufficient to retard the mobility of Chk2.

The simplest explanation for these results is that an endogenous Chk2 activating kinase is present in this system, with likely candidates being endogenous PIKs and/or Chk2 (see below). Pre-incubation of RL with 1 mM caffeine or 5 uM wortmannin inhibited phosphorylation at T26/S28 and at T68 (Fig. 2B, lanes 3 and 7). At these concentrations, caffeine inhibits human Atm and, partially, Atr, but not DNA-PK; and

wortmannin inhibits Atm and DNA-PK, but not Atr (39, 40). Loss of phosphorylation at these sites was accompanied by loss of Chk2 autophosphorylation activity assayed by ^{32}P incorporation (Fig. 2B, lanes 3 and 7). We conclude that Chk2 derived from the RL is phosphorylated by an Atm-like kinase, and that this phosphorylation is required for strong Chk2 kinase activity.

In contrast to material produced in RL, HA-Chk2 derived by translation in wheat germ extracts was not hyperphosphorylated, lacked T26/S28 and T68 phosphorylation, and had minimal auto-phosphorylation activity (Fig. 2B, lane 14, Fig. 2C, lane 2). Incubation of this material in RL enhanced T68 phosphorylation, and Chk2 autophosphorylation (Fig. 2C, lane 3; compare to Chk2 produced in RL in lane 5). To our knowledge, this is the first mammalian cell-free system to enable catalytic activation of Chk2.

SCD is phosphorylated by Chk2

In the RL system, and in intact cells exposed to IR, kinase-defective Chk2 is phosphorylated in *trans* at T26/S28 and T68 (Fig. 2B and other data not shown). However, full phosphorylation of Chk2 resulting in electrophoretic mobility shift requires a functional Chk2 kinase domain (Fig. 2B, lanes 2 and 12, and other data not shown). This suggests that, at minimum, Chk2 activation requires successive trans-phosphorylation and autophosphorylation. Phosphorylation of Chk2 within the activation loop of the kinase domain is required for catalytic activation of Chk2 (26), and other autophosphorylation sites may also contribute to the mobility shift. We analyzed a series of bacterially-expressed GST-Chk2 alleles in order to identify domains in Chk2 that are required for kinase activity, and domains that are subject to autophosphorylation (Fig. 3).

In these assays, both autophosphorylation of Chk2, and *trans*phosphorylation of a GST-Cdc25C substrate peptide were monitored. Both putative kinase-defective alleles, GST-Chk2(D347A) and GST-Chk2(D368A), lacked kinase activity in this assay. Within the SCD, the T68A substitution slightly reduced Cdc25C- and autophosphorylation activity. A more substantial decrease was observed with GST-Chk2(7A), in which all seven SQ/TQ sites within the SCD are substituted with AQ. Deletion of the entire SCD (GST-Chk2-ΔSCD) eliminated autophosphorylation, but only moderately diminished Cdc25 peptide phosphorylation. Similar results were observed with Chk2 and Chk2 mutants produced in the RL system (data not shown). These data indicate that the SCD is required for maximal Chk2 kinase activity.

The stronger effects on autophosphorylation than on transphosphorylation suggested that the SCD contains Chk2 autophosphorylation sites, including T68. Potential phosphorylation sites within the SCD include the seven SQ/TQ sites, as well as an additional 17 serines and 4 threonines. GST-Chk2 phosphorylated GST-SCD (Fig. 4, lane 3) and SCD(7A), lacking the SQ/TQ sites, to a lesser extent (Fig. 4, lane 5), but not GST (data not shown). GST fusion protein of the Chk2 FHA domain was not phosphorylated (data not shown). Hence the SCD is evidently a target for Chk2 autophosphorylation.

FHA domain is required for autophosphorylation of Chk2

FHA domains are phosphopeptide-binding modules (13, 14, 45, 47). Deletion of the core FHA domain of Chk2 resulted in a significantly lower auto-kinase activity and diminished trans-kinase activity (Fig. 3, lane 5). Similarly, mutation of the conserved FHA domain residues NGT (GST-Chk2-NGT) abrogated kinase activity (Fig. 3, lane 6).

(However, substitution of conserved R117 did not affect kinase activity.) Similar results were obtained in the RL system (data not shown). These data suggested that the FHA domain regulates Chk2 kinase activity, consistent with earlier work showing that FHA mutations prevent Chk2 activation *in vivo* (10, 26, 51)

IR enhances Atm-dependent oligomerization of Chk2.

A yeast two-hybrid screen using yeast Rad53 as bait identified Rad53 as an interacting protein (Z Sun and DF Stern, unpublished results). Since this suggested that Rad53, as well as its human homolog Chk2, forms dimers or oligomers, we determined whether oligomers were produced in mammalian cells. In 293 cells expressing FLAG-Chk2 and HA-Chk2, the tagged proteins co-immunoprecipitated, and the co-immunoprecipitation was enhanced with exposure to IR (Figs. 5A, 5B). In Atm-deficient fibroblasts originating in a patient with A-T, enhancement of Chk2 oligomerization after gamma irradiation occurred with co-expression of wild type Atm, but not kinase-defective Atm (Fig. 5B). (The higher baseline oligomerization, compared to Fig. 5A probably reflects higher total expression of Chk2 with transient expression of both tagged proteins.) These results demonstrated that at least two Chk2 molecules are components of an oligomer *in vivo*; that oligomerization is enhanced in response to DNA damage; and this enhancement is *ATM*-dependent. Thus, Chk2 oligomerization may be a regulated process that is linked to Chk2 activation.

Direct interactions of Chk2.

Co-immunoprecipitation of two tagged Chk2 molecules may occur if they form homodimers or participate in a larger complex containing additional proteins. Bacterially expressed GST-Chk2 and kinase-defective GST-Chk2(D347A) interacted with His-Flag-

Chk2 or His-Flag-Chk2(D347A) in GST-pull-down experiments even though no other eukaryotic proteins were present (Fig. 6A, 6B). However, His-Flag-Chk2(D347A) does not interact well with GST-Chk2(D347A) (Fig. 6B). Hence, Chk2 homomers can form, provided that at least one molecule has catalytic activity.

We next determined if bacterially produced GST-Chk2 or kinase-defective GST-Chk2(D347A) would pull down HA-tagged Chk2 stably expressed in 293 cells. Both GST-Chk2 and GST-Chk2(D347A) preferentially bound to HA-Chk2 after gamma irradiation (Fig. 6C). Similar results were obtained with both GST pull-down and co-immunoprecipitation of WT and kinase defective Chk2 in the RL system (data not shown). In these experiments, using Chk2 produced in bacteria, RL, or in mammalian cells, kinase-defective Chk2 associated more strongly with Chk2 than did wild type Chk2 (Fig. 6A, B and data not shown).

Chk2 oligomerization requires SCD and FHA domains

Domains required for Chk2 oligomerization were localized by mutational analysis. We first mapped the Chk2 interacting domain(s) by coimmunoprecipitation from 293 cells transiently expressing various forms of HA-tagged and Flag-tagged Chk2. Deletion of the FHA domain virtually eliminated co-immunoprecipitation of Chk2 molecules. Phosphorylation at T68 is induced by Atm and possibly Atr after DNA damage and is required for quantitative activation of Chk2 by IR (1, 33, 53). Substitution of T68 with alanine only slightly reduced oligomerization both before and after gamma irradiation (Fig. 7A). Point substitution of other SQ/TQ sites (S19, T26/S28, S33/S35, S50) did not significantly affect oligomerization (data not shown). However, mutation of all seven SQ/TQ sites including T68 within the SCD significantly diminished

coimmunoprecipitation (Fig. 7A). This indicates that Chk2 oligomerization requires one or more intact SQ/TQ sites within the SCD. Deletion and substitution mutants of bacterially produced GST-Chk2 fusion proteins were used to determine the minimal fragment of Chk2 required for binding wild type Chk2 produced in irradiated 293 cells (Fig. 7C). GST-HAFHA was sufficient to preferentially bind wild type Chk2 after DNA damage.

Since co-immunoprecipitation of two tagged Chk2 molecules required both the FHA phosphopeptide recognition domain and phosphorylation within the SCD, and since GST-FHA is sufficient to isolate Chk2 after DNA damage, we hypothesized that oligomerization of Chk2 is mediated by FHA/phospho-SCD interactions. Consistent with this hypothesis, GST-HAFHA failed to pull down HA-Chk2ΔSCD in 293 cells after gamma irradiation. GST-HAFHA did bind to HA-Chk2-SCD(7A) expressed in 293 cells after gamma irradiation (Fig. 7D), perhaps reflecting Chk2 autophosphorylation at non-SQ/TQ sites within the SCD (Fig. 4).

If an FHA domain in one Chk2 molecule associates with the phospho-SCD in another Chk2 molecule, then it should be possible to form a heterodimer containing one molecule with intact phospho-SCD, but deleted FHA domain, and another with deleted SCD, but intact FHA. Flag-Chk2ΔSCD co-immunoprecipitates with HA-Chk2-ΔFHA, and the complex is enhanced by IR (Fig. 7B).

Chk2 oligomerization is phosphorylation-dependent

In order to directly test the significance of phosphorylation on the putative FHA/phospho-SCD interaction, we determined whether the interaction is affected by phosphatase treatment. The ability of bacterially-expressed of GST-Chk2(D347A) to pull

down bacterially produced His-Flag-Chk2 (Fig. 8A) was prevented by prior dephosphorylation of His-Flag-Chk2 using λ phosphatase (Fig. 8B). Similar results were obtained using the GST-FHA domain, rather than full-length Chk2 as a pull-down reagent (Fig. 8B). In order to verify that this binding is mediated by the FHA domain, rather than other sequences present in the fusion protein, we used GST fusion proteins containing and lacking the FHA domain, and also GST1-221NGT, with alanine substitutions in a tripeptide important for FHA function. GST-Chk2 fusion proteins containing an intact FHA strongly bound to His-Flag-Chk2 but not His-Flag-Chk2(D347A). However, GST1-221NGT did not bind (Fig. 8C).

Transphosphorylation in the Chk2-Chk2 complex

An important function of Chk2 oligomerization may be that it enables cross-phosphorylation of Chk2 molecules, which, in turn, enhances Chk2 activation. Hence we determined whether Chk2 could cross-phosphorylate a second (kinase-defective) Chk2 molecule in a heterodimer. *In vitro* kinase assays using bacterially produced GST-Chk2(D347A) and His-Flag-Chk2 revealed that cross-phosphorylation of kinase-defective GST-Chk2(D347A) by His-Flag-Chk2 occurs at T68 (Fig. 6B).

Discussion

We have demonstrated that Chk2 can be catalytically activated in RL by a process that involves transphosphorylation by a wortmannin-sensitive kinase, and that full activation of Chk2 as measured by mobility shift requires a functional Chk2 catalytic domain. Maximal catalytic activity of bacterially expressed Chk2 is significantly impaired by deletion or mutation of the SCD and FHA domain. The SCD contains multiple phosphorylation sites, including a cluster of target sites for upstream regulator kinases Atm and Atr. Mutation of T68 is known to interfere with damage-dependent Chk2 responses *in vivo* (1, 33, 53). We found, as reported earlier (26), that the major Atm target T68 is also a substrate for Chk2 produced in bacteria. Additional sites, including T26S28 and site(s) besides the SQ/TQ PIK consensus are also phosphorylated by Chk2 *in vitro*. Two or more molecules of *S.c.* Rad53 or *H.s.* Chk2 are components of protein complexes. Formation of Chk2-containing complexes is enhanced by IR, requires the SCD and FHA domains, and, *in vitro*, requires phosphorylation of Chk2. Hence intra- or intermolecular interaction of Chk2 and phospho-SCD domains appears to be important in activation of Chk2.

Activation of Chk2 through Atm and/or Atr is accompanied by phosphorylation of Chk2 at multiple sites within the SCD, including the major site at T68 (1, 32, 33, 53), but phosphorylation at these sites is not sufficient for electrophoretic mobility shift in our assays (Fig. 2 and other data not shown). These same sites are also phosphorylated by recombinant Atm or Atr *in vitro* (32), and mutation of T68 diminishes *in vivo* activation and functionality of Chk2 (1, 33, 53). Despite this evidence indicating that Atm and Atr

activate Chk2 in a simple protein kinase cascade, attempts to activate Chk2 *in vitro* by phosphorylation with these kinases have not yet succeeded.

In budding yeast, additional molecules besides the PIKs Mec1 and Tel1 are required for activation of Rad53. Pie1/Ddc2/Lcd1 forms a stable complex with Mec1, and may be important for targeting Mec1 to DNA lesions or substrates (34, 36, 50). A second molecule, Rad9, is also required for damage-dependent activation of Rad53 in G2 (38, 46, 47). Rad9 is an oligomeric protein that contains two BRCT repeats (44). Rad9 is phosphorylated by Mec1 in response to DNA damage (38, 46). The phosphopeptides so created recruit Rad53 to a complex with Rad9, which is mediated by binding of Rad53 FHA domains (47). This system permits Mec1 and/or Tel1-dependent, phosphorylation-dependent binding of Rad53 to Rad9, which correlates with Rad53 activation.

Two models have been proposed for activation of Rad53 through binding of Rad9; but these models are not mutually exclusive. The first model is that phospho-Rad9 serves as a damage-dependent adapter that recruits Rad53 to Mec1, so that Mec1 can activate Rad53 by phosphorylating it at an SQ/TQ-rich cluster similar to the SCD (47). In this model, Mec1 phosphorylates both Rad9 and Rad53 directly. A second model is based upon the finding that Rad9 complexes promote ATP-dependent activation of Rad53, even when these complexes are purified from cells lacking Mec1 and Tel1 (17). Here, it is proposed that phospho-Rad9 acts as a scaffold that concentrates Rad53 and enables cross-phosphorylation and activation of Rad53 . Although this model does not require that the PIKs directly phosphorylate Rad53, such phosphorylation does seem likely since Rad53 has an SQ/TQ cluster homologous to the PIK target domain on Chk2.

In both of these models, there is an obligatory role for recruitment of Rad53 to phospho-Rad9 via the FHA domains. Mutation of the FHA domains prevents damage-induced association of Rad53 with phospho-Rad9, and concomitantly inhibits Rad53 hyperphosphorylation and efferent functions coupled through Rad9 (47). Similar effects are seen with mutation of Rad9 phosphorylation sites that are recognized by the Rad53 FHA domains (M Schwartz and DF Stern, submitted for publication).

Mutations in the Chk2 FHA domain have been found in *TP53*+/+ families with variant Li-Fraumeni syndrome. Similar to the Rad53 FHA domain, Chk2 FHA is required for PIK-dependent phosphorylation. The FHA domain of Chk2 is required for IR-induced T68 phosphorylation. This suggests that the FHA domain couples Chk2 to an Atm complex. Nevertheless, we observed that deletion of the core FHA domain of Chk2 spared T68 phosphorylation after gamma irradiation (Fig. 7E), and bound to bacterially produced FHA domain (Fig. 7D, second panel). This suggests that an additional mechanism of T68 phosphorylation bypassing the requirement of the FHA domain of Chk2 can operate under some circumstances.

Our data suggest that regulation of Chk2 by trans and autophosphorylation is more complicated than hitherto appreciated, and involves a cascade of phosphorylations possibly leading to production of Chk2 homodimers. As reported earlier, activation of Chk2 probably requires transphosphorylation by PIKs followed by autophosphorylation (26). We have identified SQ/TQ and probably non-SQ/TQ sites within the SCD as important targets for auto-as well as transphosphorylation. Phosphorylation of the SCD enables recruitment of Chk2 to homomeric or heteromeric complexes in a process that requires the FHA domain.

By analogy to the Rad9/Rad53 system, the first step in activation of Chk2 may be association of the FHA domain with a Rad9-like protein that has been phosphorylated by Atm or Atr. Since Rad9 itself oligomerizes (44), such a protein may promote Chk2 activation by concentrating Chk2 to enable cross-phosphorylation. In mammals, a functional homolog for budding yeast Rad9 has not been established. The major structural similarity between *RAD9* and its homolog *crb2* in *S. pombe* (37) lies in the tandem BRCT repeats. Brca1 is one of the few human proteins that have similar carboxyl-terminal tandem BRCT repeats. Brca1 is hyperphosphorylated after DNA damage, and this regulation requires Atm (12). Brca1 is also phosphorylated by Chk2 (27). Although these results are consistent with a Rad9-like function for Brca1, there are significant differences. Chk2 is basally associated with Brca1 (27), whereas Rad9/Rad53 interactions are promoted by DNA damage (47). Also, outside of S phase, activation of Rad53 requires *RAD9* (47), whereas Brca1 is not required for damage-dependent activation of Chk2 (31) (X Xu and DF Stern, unpublished data). However, this may simply indicate greater redundancy in Chk2 activation by DNA damage.

If the damage-dependent oligomerization of Chk2 in *in vivo* co-immunoprecipitation and pull-down experiments (Figs. 5 and 6), is mediated by association with a Rad9-like protein, the initial requirement for the FHA domain would be association with the Rad9 homolog. However, in this scenario, the requirement for the SCD is puzzling. Since bacterially-expressed Chk2 can interact with itself after phosphorylation, it is possible that recruitment and activation of a pair of Chk2 molecules by the putative Rad9 homolog results in further polymerization of Chk2 on the scaffold through phospho-SCD/FHA interactions of Chk2 molecules. This would result in

concentration of Chk2 at the site of damage, and amplification of the checkpoint signal. Indeed, there is no reason why such amplification would require a Rad9-like protein at all: once priming activation by the scaffold occurs, dimeric or polymeric Chk2 homodimers could dissociate from the Rad9-like molecule, and diffuse elsewhere to target substrates.

An important implication of this model is that phospho-Chk2 can activate other molecules of Chk2. DNA damage-dependent activation of PIKs would result in phosphorylation of Chk2 at T68 and other SQ/TQ sites within the SCD (1, 32, 33, 53). Phosphorylation of T68 would enable Chk2 autophosphorylation at the activation loop (26) and further sites within the SCD. Non-phosphorylated Chk2 molecules would then be recruited and activated through FHA/SCD interactions and cross-phosphorylation. Hence, after priming phosphorylation by PIKs, additional molecules could be activated independent of PIK activity, thereby latching on Chk2 activation.

Assuming that a Rad9-like molecule exists, then Chk2-Chk2 interactions may indirectly stabilize the scaffold/Chk2 complex, and facilitate recruitment and activation of additional Chk2 molecules. Activation of Rad53 by Rad9 purified from yeast cells is an ATP-dependent process (17). Since this process does not require Mec1 or Tel1, the simplest explanation for the ATP dependence is Rad53-dependent phosphorylation of Rad53.

Co-immunoprecipitation and GST-pulldown experiments using Chk2 derived from bacteria, RL, or 293 cells, generally demonstrated a strong preference that one, but not both, of the Chk2 molecules in the complex be catalytically active. Moreover, these experiments revealed greater recovery of heteromers containing one kinase-defective

molecule. Results of bacterial expression experiments suggest a strong preference for association of kinase-defective with kinase-active Chk2. This may reflect conformational effects of Chk2 phosphorylation on inter-Chk2 interactions. For instance non-phosphorylated Chk2 may have a more "open" conformation than phosphorylated Chk2 if an intramolecular interaction occurs upon Chk2 phosphorylation that occludes the SCD or FHA domain. Another explanation is that the kinase defect hampers dissociation of an activated complex. In this regard it is noteworthy that kinase-defective Chk2 does not dissociate from the candidate Rad9 ortholog Brca1 after DNA damage (27). We have similarly observed in co-immunoprecipitation experiments that more phospho-Rad9 is recovered in complex with Rad53 than with kinase-defective Rad53 (M. Schwartz and DF Stern, unpublished data). Three different FHA interactions may be involved: Phospho-Rad9-equivalent protein to Chk2 FHA; phospho-Chk2 to Chk2 FHA on a second molecule, phospho-Chk2 to Chk2 FHA on the same molecule. Differential stability of such complexes would produce a dynamic mechanism for assembly and disassembly of activation complexes. For example, if we postulate that phospho-Rad9-equivalent/FHA is weaker than phospho-Chk2/Chk2, then priming phosphorylation of Rad9 will permit P-Rad9/Chk2 interactions; this in turn would permit Chk2 activation and cross-phosphorylation, which would allow formation of the more stable Chk2/P-Chk2 complexes. Greater stability of intramolecular phospho-SCD/FHA than intermolecular phospho-SCD/FHA would favor disassembly of the homodimer into monomeric activated Chk2.

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Figure legends

Fig. 1. Schematic diagrams of Chk2 and Chk2 mutants. SCD, FHA, and kinase catalytic domains are marked, with amino acid coordinates above. CΔ1 corresponds to a spontaneous variant of *CHK2* from Li-Fraumeni syndrome (LFS), with frameshift mutation causing readthrough into alternate reading frames as indicated. CΔ2, a similar frameshift mutation thought to be a spontaneous variant of *CHK2* from LFS, was found to be a polymorphism in the homologous fragment present on chromosome 15 (43). KD is kinase-defective owing to substitutions in the catalytic domain. R117A, and NGT/AAA have substitutions at conserved FHA domain residues. 7A indicates substitution of S or T with A in all SQ and TQ sites within the SCD.

Fig. 2. Cell-free phosphorylation and activation of Chk2. HA-Chk2 or kinase-defective HA-Chk2(D347A) was produced by coupled transcription/translation in reticulocyte lysate (RL) or wheat germ (WL) and isolated by immunoprecipitation with anti-HA.

Panel A. Phosphorylation of HA-Chk2 produced *in vitro*. HA-Chk2 or HA-Chk2(D347A) produced in RL was immunoprecipitated and detected by immunoblotting with anti-HA. For phosphatase experiments (right lanes), immunoprecipitated HA-Chk2 was incubated in CIP buffer, or CIP buffer plus CIP at 37^0C for 1 hr.

Panel B. Chk2 phosphorylation and kinase activity. Forms of HA-Chk2 produced by cell-free transcription/translation were immunoprecipitated and incubated with [γ-^{32}P]ATP for *in vitro* kinase assays. Recovery of Chk2 was monitored by immunoblotting with anti-HA (bottom). Phosphorylation at T68, or T26/T28 was

measured by immunoblotting with the appropriate phospho-specific antibody; *in vitro* kinase activity was monitored by incorporation of [γ-^{32}P]ATP (top). ^{32}P, pT68, and HA panels are all from the same filter probed with anti-PT68, stripped, and reprobed with anti-HA. pT26/S28 are from an independent blot of portions of the same samples. In lanes 2 through 8, forms of HA-Chk2 were produced in the RL system after pre-incubation of the lysate with the designated concentration of vehicle control, caffeine, or wortmannin. In lanes 9,10,11, *in vitro* kinase assays were performed on the anti-HA immunoprecipitates in the presence of [γ-^{32}P]ATP only (lane 9) or in the presence of [γ-^{32}P]ATP and caffeine (10 mM, lane 10) or wortmannin (10 uM, lane 11).

Panel C. Activation of Chk2 produced in wheat germ lysates. HA-Chk2 was produced in the wheat germ extract system (lanes 1-3) or reticulocyte lysate system (lanes 4 and 5). HA-Chk2 was then precipitated with anti-HA antibody (lanes 2, 3, and 5) or mouse IgG (lanes 1 and 5). One Chk2 precipitate from the wheat germ extract system (lane 3) was incubated with reticulocyte lysate at 30^{0}C for 30 minutes prior to the kinase assay. All the immunocomplexes were incubated with [γ-^{32}P]ATP for *in vitro* kinase assays. Recovery of Chk2 was monitored by immunoblotting with anti-HA (bottom); with anti-pT68 (middle), or incorporation of [γ-^{32}P]ATP (top).

Fig. 3. Mutations in either SCD or FHA domain impair Chk2 kinase activities. GST-fusion proteins were produced in bacteria, purified on glutathione beads and released in soluble form with reduced glutathione. *In vitro* kinase assays were performed in the presence of Chk2 substrate GST-Cdc25C (200-256 aa) and [γ-^{32}P]ATP. ^{32}P incorporation into GST-Chk2 and mutants, upper panel; and GST-Cdc25C, second panel. GST-Chk2 fusion proteins were quantified by immunblotting with anti-Chk2

antibody (N-17), third panel. Coomassie brilliant blue staining showing substrate GST-Cdc25C (bottom panel).

Fig. 4 Chk2 phosphorylates the SCD *in vitro*. *In vitro* kinase assays were performed on soluble GST-Chk2 and mutants in the presence of substrate GST-HASCD or GST-HASCD(7A) and [γ-^{32}P]ATP. Top panel shows ^{32}P incorporation into GST-HASCD or GST-HASCD(7A). GST-HASCD or GST-HASCD(7A) (middle panel), GST-Chk2 and it mutants (bottom panel) were detected by immunoblotting with anti-GST.

Fig. 5 Chk2 oligomerization.

Panel A. 293 cells stably expressing Flag-Chk2 were transiently transfected with HA-Chk2. Transfectants were treated with 1 mM of HU for 24 hours, beginning twenty-four hours after transfection, or were exposed to gamma irradiation (20 grays), or UV (50 J/m2) 48 hours after transfection. Cell lysates were harvested 24 hours after HU treatment or 2 hours after irradiation. Upper panel, expression and mobility shift of HA-Chk2 were determined by immunoblotting lysates with anti-HA antibody. Lower panel, co-immunoprecipitation with anti-Flag antibody was performed with equal portions of cell lysates, and detected with anti-HA.

Panel B. Atm-dependent increase in Chk2 co-immunoprecipitation. GM5849C A-T cells were transiently transfected with HA-Chk2 and Flag-Chk2, plus vector (pcDNA3), wild type Flag-ATM, or kinase-defective Flag-ATMkd. Transfectants were exposed to 20 grays of gamma irradiation 48 hours after transfection. Cell lysates were harvested 2 hours after irradiation. Equal amounts of lysates were immunoprecipitated with anti-HA (top and bottom panels) or anti-Flag antibodies

(middle panel). Precipitates were blotted for Flag-Chk2 (top two panels) or HA-Chk2 (bottom panel).

Fig. 6. Oligomerization of Chk2 produced in bacteria and 293 cells.

Panel A. Bacterial expression. GST-fusion protein pull-down experiment using bacterially produced soluble GST-HAFHA, GST-Chk2 or kinase-defective GST-Chk2(D347A) incubated with His-Flag-Chk2. The pull-downs were blotted for wild type His-Flag-Chk2 with anti-Flag antibody (top panel), and for total input of GST fusions with anti-GST antibodies (bottom panel). Images of different-sized GST fusion proteins in bottom panel were cropped from one autoradiograph and aligned with one another to save space.

Panel B. Phosphorylation of Chk2D347A by Chk2. Soluble GST-Chk2D347A produced in bacteria was used to pull down soluble His-Flag-Chk2 (lanes 4, 5, 6) or kinase-defective His-Flag-Chk2(D347A) (lane 3). The affinity complexes were incubated in 1X kinase buffer in the absence (lane 5) or in the presence (lane 6) of ATP. Chk2 phosphorylation was evaluated by immunoblotting with anti-pT68. GST-Chk2D347A is significantly larger than His-Flag-Chk2.

Panel C. Mammalian expression. 293 cells stably expressing HA-Chk2 were exposed to 20 gray gamma irradiation. Cell lysates were harvested 2 hours after irradiation. Lysates were normalized for protein concentration and used for pull-down with GST-Chk2 and its mutants. The GST pull-downs were blotted for HA-Chk2 using anti-HA antibody, and for GST fusion protein using anti-Chk2 antibodies (N-17). Different sizes of GST fusions on bottom panel were cropped and realigned from a single autoradiograph.

Fig. 7 Chk2 oligomerization domains.

Panel A. Requirements for oligomerization in 293 cells. HA-tagged and Flag-tagged versions of individual Chk2 mutants were expressed by transiently transfection in 293 cells. Transfectants were exposed to 20-gray gamma irradiation 48 hours after transfection. Cell lysates were harvested 2 hours after irradiation and equal amounts were used for immunoprecipitation using anti-HA antibody (top panel) or anti-Flag antibody (bottom panel). Because homologous immunoprecipitations (such as IP anti-Flag, blot anti-Flag) were more efficient than heterologous co-immunoprecipitations (e.g. IP anti-Flag, blot anti-HA), only one-fifth equivalent of homologous immunoprecipitation samples was analyzed relative to the cross-immunoprecipitation samples.

Panel B. Oligomerization of FHA and SCD domains *in vivo*. Performed as in 7A, except that Flag-tagged and HA-tagged Chk2 molecules were evaluated in pairwise combinations listed at left.

Panel C. GST-FHA binds to activated Chk2. Various Chk2 GST-fusion proteins expressed in bacteria were used to isolate HA-Chk2 stably expressed in 293 cells. Experiments were performed as described in the legend to Panel A. Equal portions of lysate from non-irradiated and irradiated cells were incubated with GST-Chk2 and mutants. Pull-downs were blotted for HA-Chk2 using anti-HA antibody, and for input of GST fusion protein using anti-GST antibodies. Different sizes of GST fusions on bottom panel were cropped and realigned from one autoradiograph. Total lysates used for anti-HA immunoprecipitation was one-fifth of that for GST pull-down.

Panel D. Bacterially produced FHA domain of Chk2 binds to SCD in HA-Chk2 and its mutants expressed in 293 cells after gamma irradiation. Same strategy

described previously was used. Only one representative immunoblot for input of GST fusions (bottom panel) was shown. Different sizes of GST fusions on bottom panel were cropped from one autoradiograph.

Panel E. Thr68 phosphorylation of Chk2 and its mutants *in vivo*. Cells were handled essentially as described in Panel A. Cell lysates were immunoprecipitated with anti-HA, and detected by immunoblotting with anti-pT68 or anti-HA

Fig. 8. Phosphorylation-dependent oligomerization of Chk2.

Panel A. Bacterially expressed His-Flag-Chk2 was incubated in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of λ phosphatase, and, either blotted directly with anti-pT68 or anti-Flag (lanes 5 and 6), or pulled down using either GST, or GST-Chk2(D347A) expressed in bacteria (lanes 1,2,3,4). Affinity-isolated Chk2 was detected by blotting with anti-Flag, and loading of GST-proteins was monitored with anti-GST.

Panel B. Phosphorylation-dependent binding of Chk2 FHA domain. Bacterially expressed His-Flag-Chk2 or His-Flag-Chk2(D347A) was incubated in the absence (lanes 5, 6, 11) or presence (lanes 7, 8, 12) of λ phosphatase, and immunoblotted with anti-Flag or anti-pT68 (lanes 9, 10, 11, 12). Bacterially-produced GST or GST-HAFHA was used to pull down additional portions of phosphatase-treated or non-treated kinase-defective His-Flag-Chk2(D347A) or His-Flag-Chk2. Wild-type and kinase-defective His-Flag-Chk2 were detected using anti-Flag antibody (upper panel), for input of GST-HAFHA using anti-HA antibody (second panel), and for GST using anti-GST antibody (lower panel).

Panel C. Effect of FHA mutations on binding to His-Flag-Chk2. GST-fusion proteins expressed in bacteria were used to pull down bacterially produced wild type and

kinase-defective His-Flag-Chk2. His-Flag-tagged Chk2 was detected using anti-Flag antibody, and GST fusions using anti-GST. Differently sized GST fusion proteins on the bottom panel were cropped and realigned from one autoradiograph.

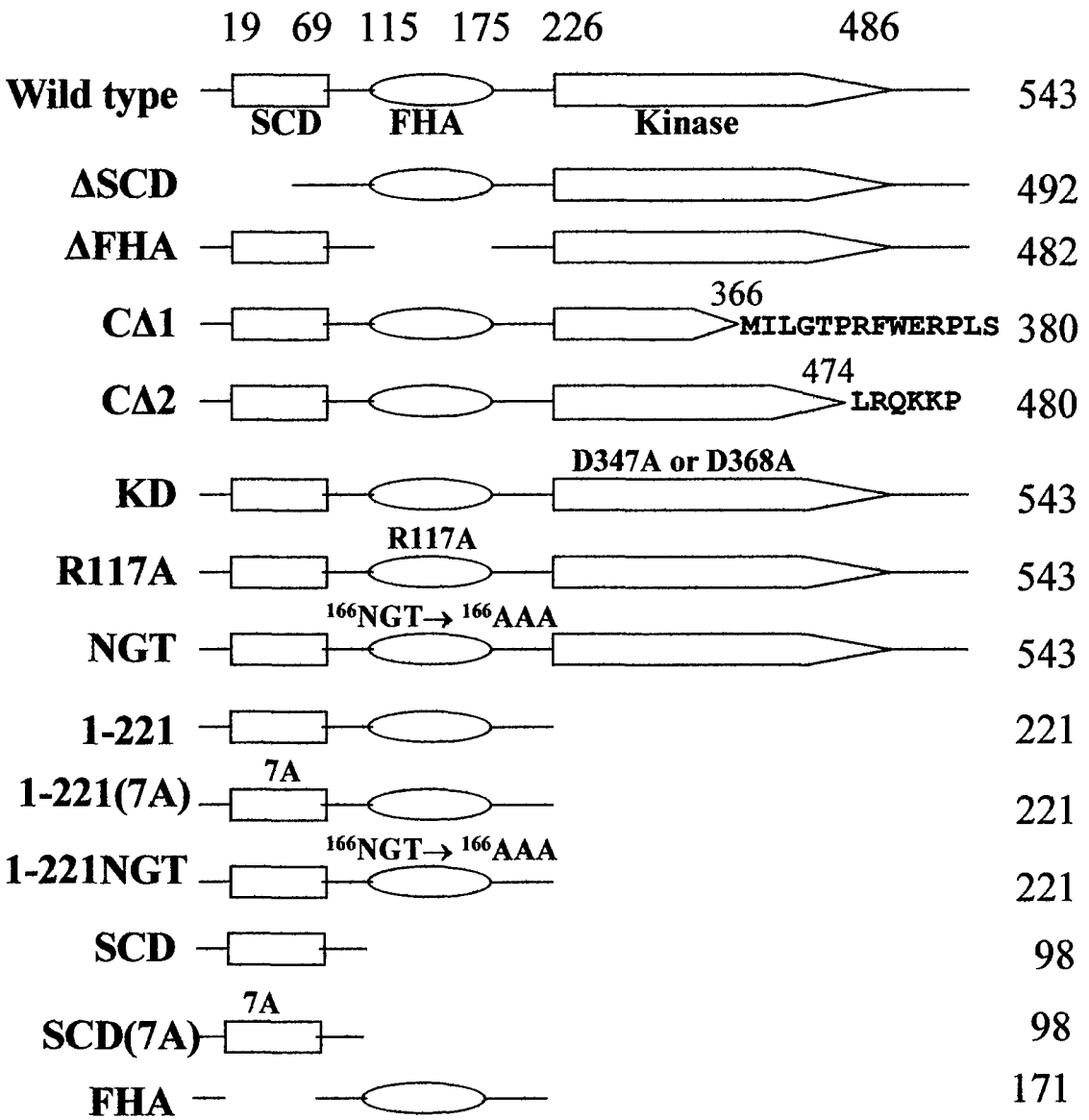

Fig. 1

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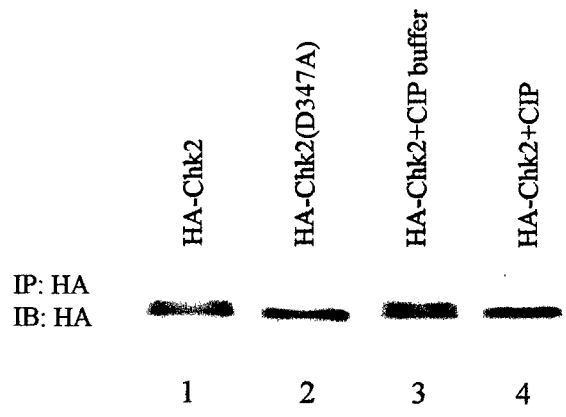

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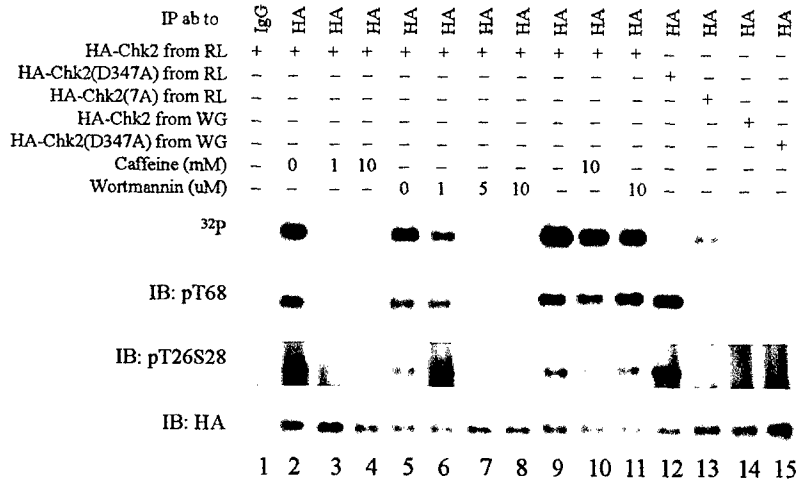

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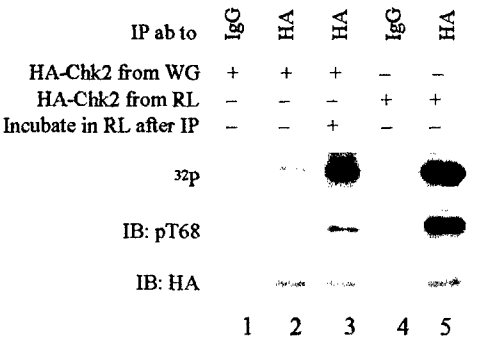

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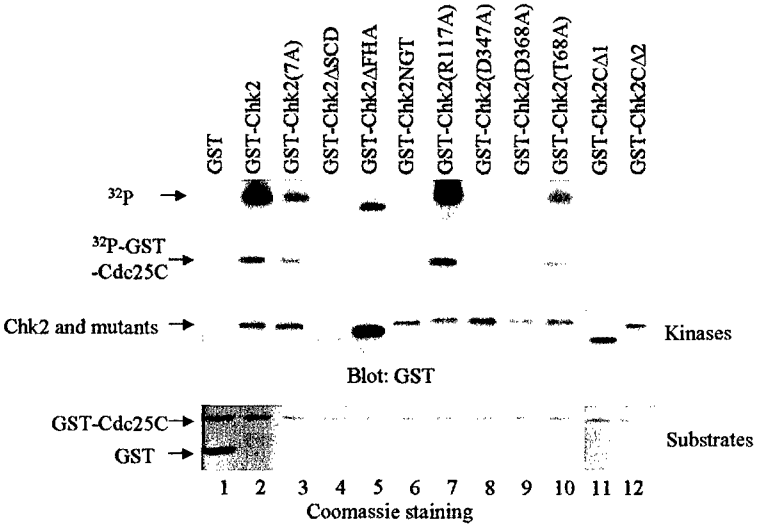

Fig. 3
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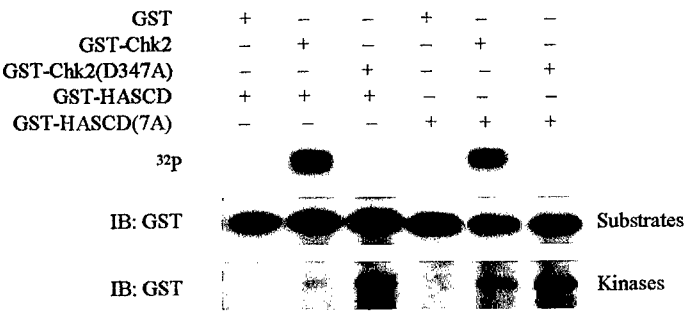

Fig. 4
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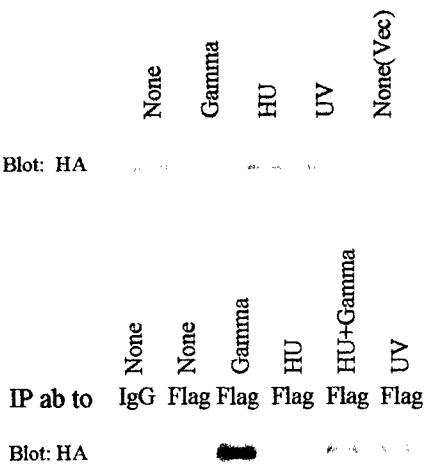

Fig. 5A
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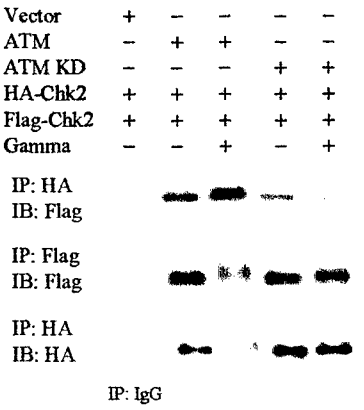

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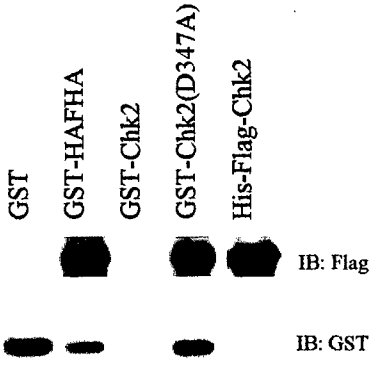

Fig. 6A
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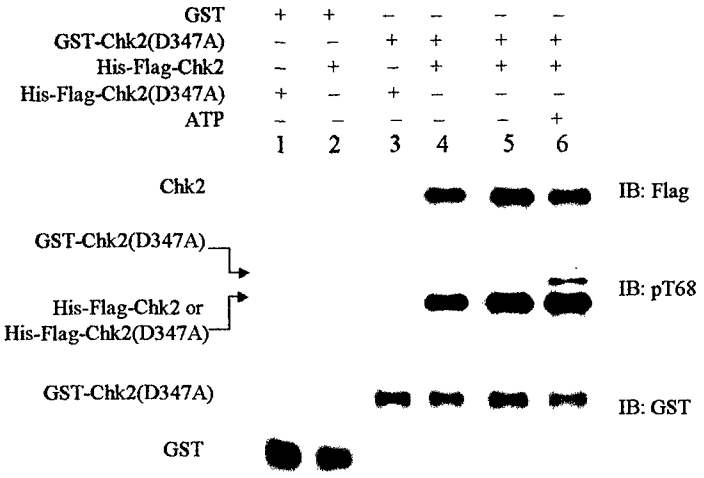

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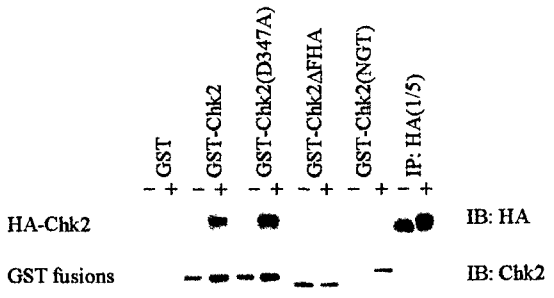

Fig. 6C
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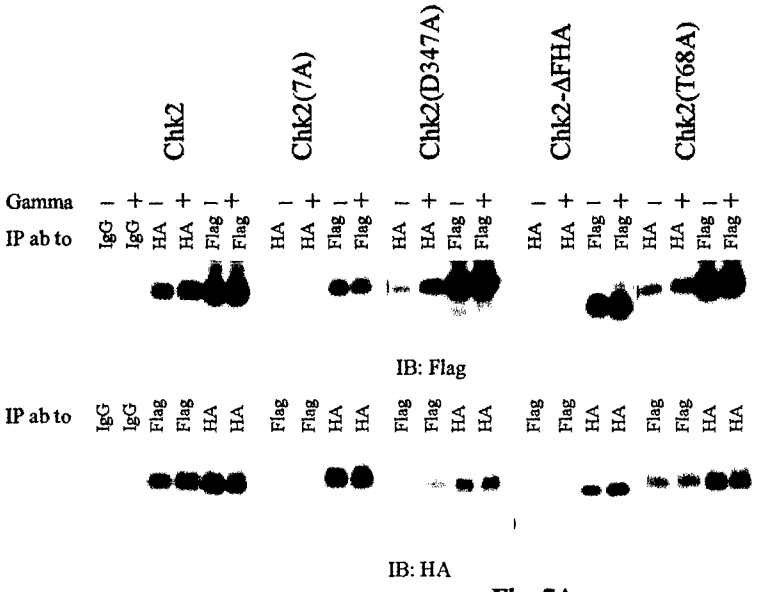

Fig. 7A
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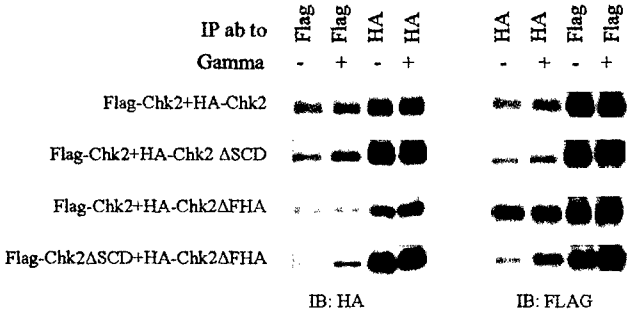

Fig. 7B
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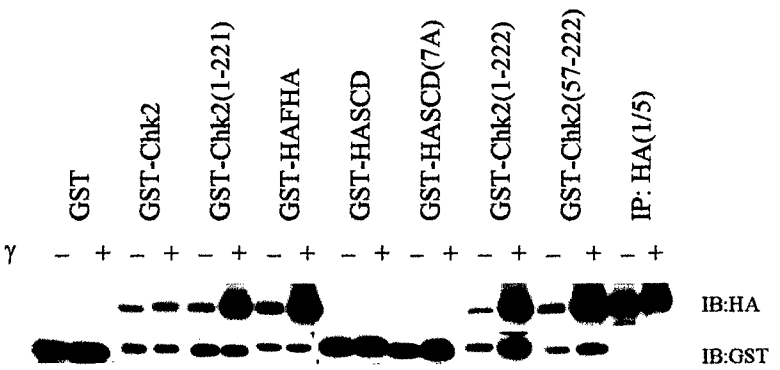

Fig. 7C
X Xu, LM Tsvetkov, and DF Stern.

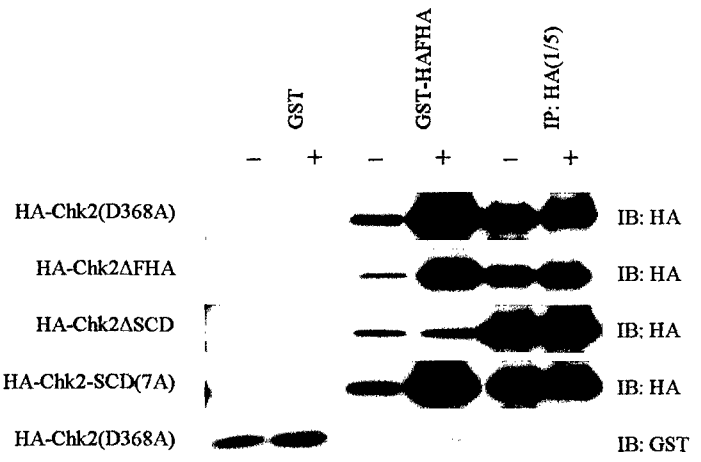

Fig. 7D
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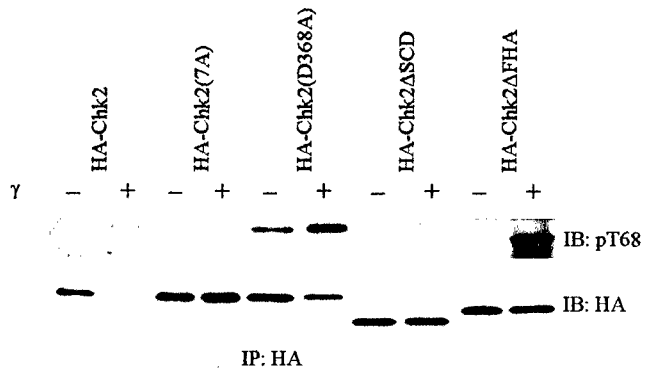

Fig. 7E
X Xu, LM Tsvetkov, and DF Stern.

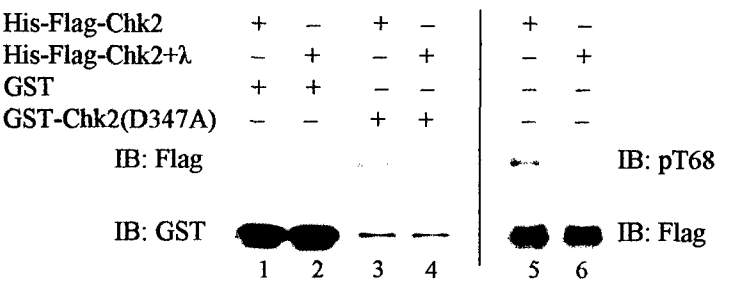

Fig. 8A
X Xu, LM Tsvetkov, and DF Stern.

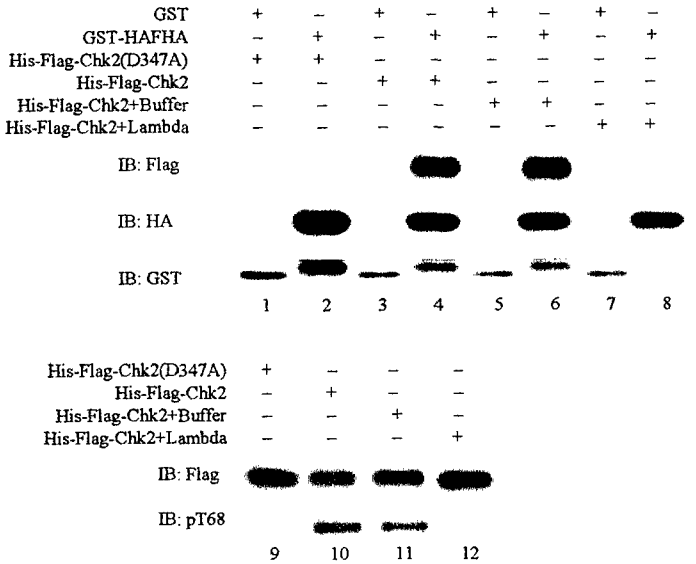

Fig. 8B
X Xu, LM Tsvetkov, and DF Stern.

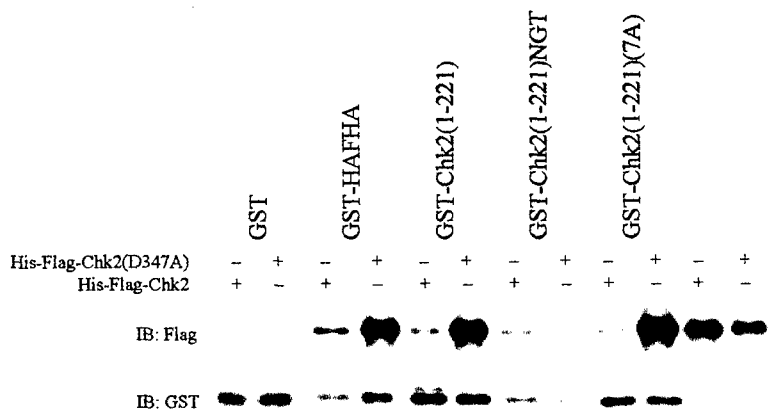

Fig. 8C
X Xu, LM Tsvetkov, and DF Stern.